  Bull. Chem. Soc. Jpn., 57, 2156—2161 (1984) [Vol. 57, No. 8

Phosphinyl- and Phosphinothioylamino Acids and Peptides. VIII. New Practical Removal Conditions for the *S*-Mpt Group and their Application for the Synthesis of Bis[*N,N*-diallyl-[D-Ala^2, L-Leu^5]-enkephalyl]cystine

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(Received January 10, 1984)

Synthesis of bis[*N,N*-diallyl-[D-Ala^2, L-Leu^5]-enkephalyl]cystine, which is expected to be a selective opioid δ-receptor antagonist, was studied. The mercapto group of cysteine was protected by the dimethylphosphinothioyl(Mpt) group. For the removal of this group without damaging the allyl moiety, new mild removal conditions by use of KF/18-crown-6 in a solvent mixture of acetonitrile-methanol are proposed. Bis[D-Ala^2, L-Leu^5]-enkephalyl]cystine was also prepared in a similar manner.

Since the discovery of enkephalins by Hughes and Kosterlitz,[1] chemistry and biochemistry of opioid peptides have attracted the attention of many reseachers. Many analogs and derivatives have been prepared in order to develop more potent, more selective and metabolically stable ligands and also to explain the structure-activity relationship and drug-receptor interactions.[2] Of these, *N,N*-diallyl-[L-Leu^5]-enkephalin methyl ester (ICI 139462, **1**) and its metabolically more stable analog (ICI 154129, **2**) are of special interest because they showed selective antagonistic activity toward the δ-receptor, but their affinity was not sufficiently high.[3] Another interesting recent finding is that a dimeric enkephalin **3** has increased affinity and selectivity for the δ-receptor as compared with the corresponding monomer.[4] We have tried to synthesize *N,N*-diallyl-[D-Ala^2, L-Leu^5]-enkephalin dimerized at extended *C*-terminal cystine **4**. This paper describes the synthetic procedures.

$(CH_2{=}CHCH_2)_2$L-Tyr-Gly-Gly-L-Phe-L-Leu-OMe **1**

$(CH_2{=}CHCH_2)_2$L-Tyr-Gly-$NHCH_2CH_2S$-$CH(CH_2C_6H_5)$-L-Leu-OH **2**

(H-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-NH-CH_2)$_2$ **3**

[$(CH_2{=}CHCH_2)_2$L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys(1)-OH]$_2$ **4**

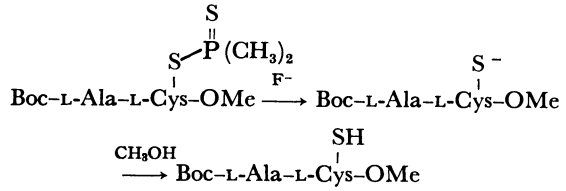
Results and Discussion

In planning the synthesis of **4**, selection of a mercapto protecting group was important. Various kinds of protecting groups for the cysteine mercapto function are now available; most of these require liquid HF or heavy metal ions for removal.[5] In the latter case, cysteine-containing peptides are generated from their thiolates by treatment with thiols. Removal conditions that may damage the double bond of the allyl group should be avoided.

Recently, we found that diphenylphosphinothioyl (Ppt)[6] and dimethylphosphinothioyl (Mpt)[7] groups, which are removed by treatment with an aqueous alkali or $AgNO_3$ solution, could be used for the protection of the mercapto group of cysteine. Independently, Horner *et al.*[8] also reported the use of the Ppt group for the same purpose and recommended its removal by unsolvated fluoride ions in dichloromethane, chloroform or tetrahydrofuran. This removal method seemed to be attractive, but it was not shown whether more polar solvents, which dissolve large peptide molecules, could also be used. We have investigated solvent effects in the removal reactions of the *S*-phosphinothioyl groups by fluoride ions.

As a preliminary experiment, a comparison was made between Mpt and Ppt groups. When 2 equivalents of tetrabutylammonium fluoride trihydrate (**5**) were added at 30 °C to a solution of Boc-L-Ala-L-Cys(Mpt)-OMe (**6**) in dichloromethane the *S*-Mpt group was cleaved instantaneously. However, removal of the *S*-Ppt group of Boc-L-Ala-L-Cys(Ppt)-OMe in the same manner took about 15 min. Based on these results, further studies were made by use of compound **6**, which carries the *S*-Mpt group. As a source of fluoride ions, KF in the presence of an equimolar amount of 18-crown-6 was also used because tetrabutylammonium fluoride trihydrate was too hygroscopic. All results are summarized in the Table 1.

The removal of the *S*-Mpt group by **5** was almost instantaneous in all aprotic solvents examined. The deprotection by KF/18-crown-6 also took place rapidly. On the contrary, the reaction in methanol was slow because of solvation of fluoride ions. Since methanol is a good solvent for large peptides, we tried the use of methanol by mixing with acetonitrile or dichloromethane and found that these mixed solvents were effective for this reaction up to the methanol content of 50%. In these solvent mixtures, no difference of cleaving abilities of tetrabutylammonium fluoride and KF-crown ether was observed. It was also noted that methanol protonated rapidly to yield the thiol form product. From these results it was concluded that the *S*-Mpt group would be useful for the synthesis of large peptides containing cysteine or cystine.



As the first application of the *S*-Mpt group involving the new removal conditions, synthesis of bis[*N,N*-diallyl-[D-Ala^2, L-Leu^5]-enkephalyl]cystine (**4**) was performed according to the scheme illustrated in Fig. 1

TABLE 1. SOLVENT EFFECTS IN REMOVAL OF *S*-PHOSPHINOTHIOYL GROUPS BY FLUORIDE IONS

Compound	Solvent	Time for complete removal (at 30 °C) KF/18-crown-6	$(n\text{-}C_4H_9)_4N^+F^-$
Boc-L-Ala-L-Cys(Ppt)-OMe	CH_2Cl_2		15 min
Boc-L-Ala-L-Cys(Mpt)-OMe (**6**)	CH_2Cl_2	4 h	Instantaneous
6	$CHCl_3$	3 h	Instantaneous
6	THF*	>4 h	Instantaneous
6	CH_3CN	30 min	Instantaneous
6	CH_3OH	4 h	4 h
6	CH_3CN-CH_3OH (8:2)	30 min	
6	CH_3CN-CH_3OH (1:1)	1 h15 min	1 h
6	CH_2Cl_2-CH_3OH (1:1)	1 h15 min	1 h
6	CH_3CN-CH_2Cl_2-CH_3OH (1:1:1)	30 min	

* Tetrahydrofuran

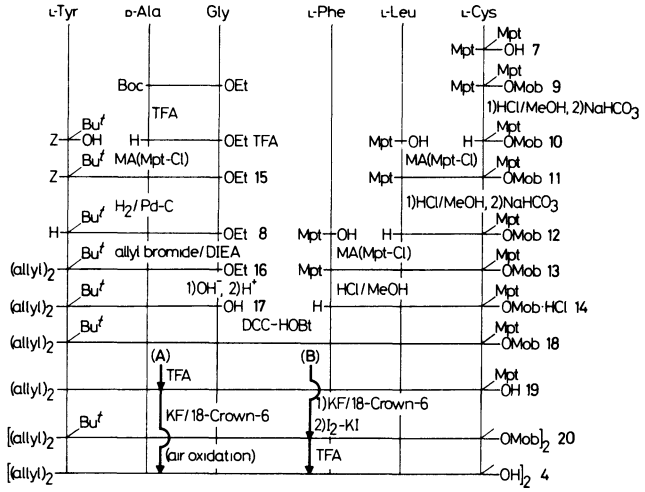


Fig. 1. Synthesis of Bis[*N*,*N*-diallyl-[D-Ala^2, L-Leu^5]-enkephalyl]cystine.

Synthesis was made by the classical method, starting with *N*,*S*-bis(Mpt)-L-cysteine (**7**).[7] **7** was esterified with *p*-methoxybenzyl alcohol in a 87% yield by use of 2 equivalents of *N*-(dimethylphosphinothioyl)imidazole.[9] From the *N*, *S*-bis-Mpt cysteine *p*-methoxybenzyl ester thus obtained, the *N*-Mpt group was selectively removed by treating with 0.4 M† HCl in methanol (3 equiv) at room temperature overnight. Succeeding couplings were performed by the Mpt-mixed anhydride method.[10] This method would allow incorporation of tyrosine moiety without protecting its phenolic hydroxyl group, but in this synthesis *t*-butyl group was introduced as a protecting group in order to avoid *O*-allylation in an *N*-allylation step. For the *N*-allylation, *N*-terminal tripeptide ester **8** was treated with excess allyl bromide in the presence of *N*,*N*-diisopropylethylamine in chloroform at room temperature for 2 d. After hydrolysis of the ethyl ester, *N*,*N*-diallyltripeptide acid **17** was coupled with *C*-terminal tripeptide ester **14** by the dicyclohexylcarbodiimide (DCC)-1-hydroxybenzotriazole (HOBt) method[11] to give protected, *N*,*N*-diallylhexapeptide ester **18** in a yield of 68%.

† 1 M=1 mol dm^{-3}.

Final deprotection required two steps: Treatment with trifluoroacetic acid (TFA) to cleave the *t*-butyl ether and *p*-methoxybenzyl ester bonds and treatment with KF/18-crown-6 to remove the *S*-Mpt group. When the *S*-Mpt group was removed at the first step, the removal conditions determined in the model experiments were effective, except that a somewhat longer reaction time (9 h) was required. After oxidation by iodine and final deprotection by TFA, the desired compound **4** was obtained. But in this case, purification by preparative thin-layer chromatography and gel chromatography in Sephadex LH-20 failed to give completely pure material, probably because of incomplete deprotection by TFA.

On the other hand, when the removal of the *S*-Mpt was performed at the final step, some difficulty was encountered, probably because the *S*-Mpt group was blocked conformationally. But when the excess reagents (10 equiv KF+4 equiv 18-crown-6) were used at higher temperature (50 °C), the removal was complete after 10 h. During these procedures, air oxidation of the product occurred to give **4** directly; this could be purified by preparative silica-gel thin-layer chromatography and gel chromatography on Sephadex LH-20.

Based on the success in the synthesis of **4**, we also prepared bis[D-Ala^2, L-Leu^5]-enkephalyl]cystine with free *N*-terminals in order to determine the effects of *N*-allylation on the pharmacological activity. In this case tyrosine was incorporated without protecting its phenolic hydroxyl group. Again, a better result was obtained when the *S*-Mpt group was removed before removal of *N*- and *C*-terminal protecting groups by TFA.

As expected, the compound **4** showed selective antagonistic activity toward the opioid δ-receptor. Additionally, the compound **4** was found to be approximately ten-fold more effective than ICI 154129 in antagonizing the δ-receptor agonists. Thus, as far as we know, the compound **4** seems to be the most potent δ antagonist.

Experimental

Thin-layer chromatography (TLC) was performed on

silica gel plates (Merck 60F_{254}) in the following solvent systems: Ether (R_f^1), ethyl acetate (R_f^2), chloroform–methanol (9:1, R_f^3), chloroform–methanol–acetic acid (95:5:3, R_f^4), chloroform–methanol–acetic acid (85:25:20, R_f^5), chloroform–methanol–water (65:25:4, R_f^6), chloroform–methanol –29% aqueous ammonia (60:30:5, R_f^7), 1-butanol–acetic acid–water (4:1:1, R_f^8).

N,S-Bis(dimethylphosphinothioyl)-L-cysteine p-Methoxybenzyl Ester (Mpt-L-Cys(Mpt)-OMob) (9). To a cooled solution of Mpt-L-Cys(Mpt)-OH[7] (1.53 g, 5 mmol) and *p*-methoxybenzyl alcohol (0.62 cm³, 5 mmol) in *N,N*-dimethylformamide (1 cm³) and chloroform (15 cm³), *N*-Mpt-imidazole (1.60 g, 10 mmol) was added; the solution was stirred at 0 °C for 4.5 h and at room temperature overnight. After removal of precipitates by filtration, the filtrate was evaporated *in vacuo*. The residue was dissolved in ethyl acetate. The solution was washed successively with water, an ice-cold 5% citric acid solution, water, a 5% sodium hydrogencarbonate solution, water, and a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated. The oily residue was dissolved in dichloromethane and placed in a silica-gel column (2.5×37 cm). Elution with ether gave **9** as colorless oil; 1.86 g (87%). $[\alpha]_D^{26}$ −5.12 ° (*c* 1, methanol); R_f^1 0.54, R_f^2 0.68. Found: C, 42.35; H, 5.93; N, 3.27; P, 14.49%. Calcd for $C_{15}H_{25}NO_3P_2S_3$: C, 42.34; H, 5.91; N, 3.29; P, 14.56%.

S-Dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester Hydrochloride (H-L-Cys(Mpt)-OMob·HCl) (10·HCl). Compound **9** (1.86 g, 4.37 mmol) was stirred with 0.4 M HCl in methanol (32.8 cm³) at 0 °C for 1 h and the mixture was kept standing at room temperature overnight. The solvent was evaporated *in vacuo* and the residue was triturated with ether. Crystalline precipitates were collected by filtration, washed with ether, and dried over P_2O_5; 1.56 g (95%). The sample was once changed to the free amine form and again precipitated from 3 M HCl in ethyl acetate (1.38 cm³); 1.17 g (72%). An analytical sample was recrystallized from hot methanol; mp 136—137 °C (decomp); $[\alpha]_D^{26}$ +3.52 ° (*c* 1, methanol); R_f^4 0.23, R_f^5 0.64. Found: C, 42.38; H, 5.72; N, 3.79; P, 8.22%. Calcd for $C_{13}H_{21}NO_3PS_2Cl$: C, 42.22; H, 5.72; N, 3.79, P, 8.37%. This salt was dissolved in water and neutralized with sodium hydrogencarbonate. The solution was extracted with ethyl acetate several times. The extracts were dried and evaporated to give the free base **10** as colorless oil.

N-Dimethylphosphinothioyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester (Mpt-L-Leu-L-Cys(Mpt)-OMob) (11). Mpt-L-Leu-OH·DCHA[12] (1.16 g, 2.87 mmol) was dissolved in chloroform (3 cm³) and treated with Mpt-Cl (0.369 g, 2.87 mmol) at 0 °C for 40 min. To this **10** (0.957 g, 2.87 mmol) and *N,N*-diisopropylethylamine (0.50 cm³, 2.87 mmol) in chloroform (3 cm³) were added and the solution was stirred at 0 °C for 1 h. After the usual washing procedures,the product was obtained by silica-gel column chromatography as a colorless oil; 1.35 g (87%). $[\alpha]_D^{25}$ −59.3 ° (*c* 1, methanol); R_f^2 0.30, R_f^3 0.67. Found: C, 46.63; H, 6.76; N, 5.15; P, 11.49%. Calcd for $C_{21}H_{36}N_2O_4P_2S_3$: C, 46.83; H, 6.74; N, 5.20; P, 11.50%.

L-Leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester (H-L-Leu-L-Cys(Mpt)-OMob) (12). The Mpt group of **11** (1.23 g, 2.28 mmmol) was removed by 0.4 M HCl in methanol (28.8 cm³). After evaporation of the solvent, ether and water were added to the residue. The ether layer was separated and washed with water. The water layer and extracts were gathered, neutralized with sodium hydrogen-carbonate and extracted with ether. The extracts were dried over anhydrous sodium sulfate and evaporated to give a colorless oil; 0.967 g (95%).

N-Dimethylphosphinothioyl-L-phenylalanyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester (Mpt-L-Phe-L-Leu-L-Cys(Mpt)-OMob) (13). Mpt-L-Phe-OH·DCHA[12] (1.035 g, 2.36 mmol) was dissolved in chloroform (5 cm³) and treated with Mpt-Cl (0.303 g, 2.36 mmol) at 0 °C for 1 h. To this a solution of **12** (1.05 g, 2.36 mmol) and *N,N*-diisopropylethylamine (0.41 cm³, 2.36 mmol) in chloroform (3 cm³) was added. The solution was stirred at 0 °C for 1 h and then evaporated *in vacuo*. After the usual washing procedures the product was obtained by silica-gel column chromatography eluted by chloroform–methanol (98:2). Pure materials were obtained by recrystallization from ethyl acetate–ether as colorless crystals; 1.30 g (79%). $[\alpha]_D^{26}$ −60.4 ° (*c* 1, methanol); R_f^1 0.18, R_f^2 0.67. Found: C, 52.21; H, 6.60; N, 6.11; P; 8.73%. Calcd for $C_{30}H_{45}N_3O_5P_2S_3 \cdot 1/2H_2O$: C, 52.08; H, 6.65; N, 6.07; P, 8.82%.

L-Phenylalanyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester Hydrochloride (H-L-Phe-L-Leu-L-Cys(Mpt)-OMob. HCl) (14). Compound **13** (1.43 g, 2.09 mmol) was stirred with 0.4 M HCl in methanol (15.7 cm³) at 0 °C for 1 h and the mixture was kept standing at room temperature overnight. The solvent was evaporated *in vacuo* and the residue was triturated with ether. Crystalline precipitates were collected by filtration, washed with ether and ethyl acetate and dried over P_2O_5; 1.089 g (82%). R_f^4 0.07, R_f^5 0.74.

N-Benzyloxycarbonyl-O-t-butyl-L-tyrosyl-D-alanylglycine Ethyl Ester (Z-L-Tyr(Bu^t)-D-Ala-Gly-OEt) (15). Mpt-Cl (0.257 g, 2 mmol) was added to an ice-cold stirred solution of Z-L-Tyr(Bu^t)-OH·DCHA[13] (1.105 g, 2 mmol) in chloroform (4 cm³), after which the mixture was stirred for 1 h. To this solution, a mixture of TFA·H-D-Ala-Gly-OEt (0.576 g, 2 mmol) and triethylamine (0.56 cm³, 4 mmol) in chloroform (2 cm³) and *N,N*-dimethylformamide (2 cm³) was added in several portions at 0 °C. After having been stirred at this temperature for 1 h, the solution was washed in the usual way, dried and evaporated *in vacuo*. The oily residue was dissolved in dichloromethane and placed in a silica-gel column (1.1×12 cm). Elution with ether gave **15** as a colorless oil; 0.899 g (85%).

N,N-Diallyl-O-t-butyl-L-tyrosyl-D-alanylglycine Ethyl Ester ((allyl)$_2$-L-Tyr(Bu^t)-D-Ala-Gly-OEt) (16). A mixture of **15** (0.899 g, 1.70 mmol), 10% palladium–charcoal (0.42 g), cyclohexene (15 cm³), and ethanol (30 cm³) was refluxed for 7 h. After removal of the catalyst by filtration the filtrate was evaporated *in vacuo*. The oily residue was dissolved in chloroform (15 cm³) and *N,N*-diisopropylethylamine (1.05 cm³, 6 mmol) and cooled to 0 °C. To this solution allyl bromide (8.65 cm³, 100 mmol) was added drop by drop over a period of 30 min at this temperature and the solution stirred at room temperature for 2 d. The solution was evaporated and the oily residue was shaken with ethyl acetate and water. The organic layer was separated and washed with water, a 5% sodium hydrogencarbonate solution, water and a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated. The oily residue was purified by preparative silica-gel thin-layer chromatography using ether as a developing solvent. From the desired band (R_f=0.4—0.6) compound **15** was obtained as colorless oil; 0.544 g (67%). An analytical sample was obtained by rechromatography on Sephadex LH-20 (1.9×100 cm) eluted with methanol. The desired fractions were collected and evaported *in vacuo* to give **16** as colorless oil; 0.544 g (67%). $[\alpha]_D^{26}$ −18.4 ° (*c* 1, methanol); R_f^2 0.45, R_f^3 0.49. Found: C, 65.92; H, 8.46; N, 8.82%. Calcd for $C_{26}H_{39}N_3O_5$: C, 65.94; H, 8.32; N, 8.87%.

N,N-Diallyl-O-t-butyl-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester ((allyl)$_2$-L-Tyr(Bu^t)-D-Ala-Gly-L-Phe-L-Leu-L-Cys(Mpt)-OMob) (18). Compound **16** (0.492 g, 1.04 mmol) was dissolved in methanol (3 cm³). To this a 1 M aqueous NaOH solution (1.23 cm³) was added at 0 °C. After the solution had been stirred at this temperature for 30 min

and at room temperature overnight, methanol was removed *in vacuo*. The resulting solution was acidified to pH 4 by acetic acid at 0 °C. To this,Diaion HP-20 resin was added until no spot corresponding to (allyl)$_2$-L-Tyr(Bu^t)-D-Ala-Gly-OH (**17**) was detectable on TLC. The resin was filtered and washed with water until the filtrate became neutral. Elution of **16** was performed by methanol to give an amorphous solid: 0.448 g (100%).

To a suspension of **17** (0.298 g, 0.669 mmol) in chloroform (2 cm^3) and dissolved *N,N*-diisopropylethylamine (0.117 cm^3, 0.669 mmol) and **14** (0.442 g, 0.669 mmol), dicyclohexylcarbodiimide (0.138 g, 0.669 mmol) and 1-hydroxybenzotriazole (0.090 g, 0.669 mmol) were added at 0 °C. The mixture was stirred at this temperature for 1 h, and at room temperature overnight. Precipitates were filtered off and the filtrate was evaporated *in vacuo*. The solid residue was dissolved in dichloromethane and washed with water, a 5% sodium hydrogencarbonate solution, water and a saturated sodium chloride solution, dried over anhydrous sodium sulfate and evaporated. Crude materials were then purified by preparative silica-gel thin-layer chromatography using chloroform–methanol (100:1) as a developing solvent system. The desired band was eluted with dichloromethane–methanol (9:1) and the eluates evaporated *in vacuo*. The residue was again dissolved in a small volume of dichloromethane and placed in a Sephadex LH-20 column (1.9×100 cm) and eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to give **18** as colorless crystals; 0.471 g (68%). An analytical sample was recrystallized from hot methanol; mp 188—190 °C; $[\alpha]_D^{26}$−20° (*c* 1, *N,N*-dimethylformamide); R_f^3 0.61, R_f^4 0.40. Found: C, 60.90; H, 7.17; N, 8.21; P, 2.63%. Calcd for $C_{52}H_{73}N_6O_9PS_2$: C, 61.16; H, 7.20; N, 8.23; P, 3.03%.

N,N-*Diallyl-*L*-tyrosyl-*D*-alanylglycyl-*L*-phenylalanyl-*L*-leucyl-S-dimethylphosphinothioyl-*L*-cysteine ((allyl)$_2$-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys(Mpt)-OH) (**19**).* Trifluoroacetic acid (4 cm^3) was added to an ice-cold solution of compound **18** (0.236 g, 0.23 mmol) in dichloromethane (4 cm^3) containing anisole (0.4 cm^3). After having been kept at room temperature for 6.5 h, the solution was evaporated *in vacuo*. The solid residue was washed with ether and dissolved in methanol (10 cm^3) and water (2 cm^3). The solution was neutralized to pH 7 by addition of solid sodium hydrogencarbonate at 0 °C, and then evaporated. The residual materials were submitted to preparative silica-gel thin-layer chromatography in a solvent system of chloroform–methanol–water (65:25:4). The desired band (R_f=0.5—0.7) was eluted with methanol and evaporated *in vacuo*. The residue was dissolved again in a small volume of methanol and placed in a Sephadex LH-20 column (1.9×100 cm) and eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to give **19** as colorless crystals; 0.175 g (87%). An analytical sample was recrystallized from hot methanol; mp 169—172 °C; $[\alpha]_D^{26}$+7 °(*c* 1, acetic acid); R_f^6 0.54, R_f^7 0.64. Found: C, 55.08; H, 6.61; N, 9.56; P, 3.14%. Calcd for $C_{40}H_{57}N_6O_8PS_2$. 4/3H_2O: C, 55.28; H, 6.92; N, 9.67; P, 3.56%.

*Bis(*N,N-*diallyl-*O*-t-*butyl-*L*-tyrosyl-*D*-alanylglycyl-*L*-phenylalanyl-*L*-leucyl)-*L*-cystine Bis(*p*-methoxybenzyl Ester) ((allyl)$_2$-L-Tyr(Bu^t)-D-Ala-Gly-L-Phe-L-Leu-L-Cys-OMob)$_2$ (**20**).*

To a solution of compound **18** (0.294 g, 0.288 mmol) in methanol (3 cm^3) and acetonitrile (3 cm^3), KF (33.5 mg, 0.576 mmol) and 18-crown-6 (0.152 g, 0.576 mmol) were added. The mixture was stirred at room temperature for 9 h and treated with 0.1 M iodine–potassium iodide in 90% methanol solution (1.6 cm^3). The yellow solution was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed with water, a sodium thiosulfate solution, water, a 5% citric acid solution, water, a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated *in vacuo*. The residue was purified by preparative silica-gel thin-layer chromatography in a solvent system of chloroform–methanol (95:5). The desired band (R_f=0.6—0.7) was eluted with methanol and evaporated *in vacuo*. The residue was again purified by gel chromatography on Sephadex LH-20 (1.9×100 cm) eluted with methanol. The desired fractions were collected and eluates evaporated *in vacuo* to yield **20** as colorless crystals; 0.237 g (88%). Mp 196—199 °C; R_f^3 0.54, R_f^4 0.29. Found: C, 64.25; H, 7.25; N, 9.00%. Calcd for $C_{100}H_{134}N_{12}O_{18}S_2 \cdot CH_3OH$: C, 64.24; H, 7.37; N, 8.90%.

*Bis(*N,N-*diallyl-*L*-tyrosyl-*D*-alanylglycyl-*L*-phenylalanycyl-*L*-leucyl)-*L*-cystine ((allyl)$_2$-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys-OH)$_2$ (**4**).* *Method A*: To a solution of compound **19** (0.129 g, 0.149 mmol) in methanol (10 cm^3) and acetonitrile (10 cm^3), KF (0.083 g, 1.48 mmol) and 18-crown-6 (0.158 g, 0.595 mmol) were added. The mixture was stirred at 50 °C for 10 h and evaporated *in vacuo*. The residue was dissolved in a small volume of methanol and water and applied to a column (1.2×21 cm) of Dowex 50 W×2 (H^+ form). The resin was rinsed with water (150 cm^3) and then the desired material was eluted with a 1% aqueous ammonia solution. The crude peptide was purified by preparative silica-gel thin-layer chromatography in a solvent system of chloroform–methanol–water (65:25:4) and gel chromatography on Sephadex LH-20 (1.9×100 cm) eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to yield **4** as colorless crystals; 58 mg (51%). An analytical sample was further purified by preparative silica-gel thin-layer chromatography in a solvent system of 1-butanol–acetic acid–water (4:1:1) and gel chromatography on Sephadex LH-20. Mp 198—208 °C (decomp); $[\alpha]_D^{26}$+6.7 (*c* 1, acetic acid); R_f^6 0.20, R_f^7 0.21, R_f^8 0.52. Found: C, 56.87; H, 6.67; N, 10.17%. Calcd for $C_{76}H_{102}N_{12}O_{16}S_2 \cdot 2H_2CO_3 \cdot H_2O$: C, 56.92; H, 6.61; N, 10.21%.

Method B: Compound **20** (0.236 g, 0.127 mmol) was stirred with trifluoroacetic acid (4 cm^3) containing anisole (0.8 cm^3) at 0 °C for 1 h and at room temperature over night. The solution was evaporated *in vacuo* and the residue was crystallized by triturating with ether. Crystalline precipitates were purified by preparative silica-gel thin-layer chromatopraphy using chloroform–methanol–29% aqueous ammonia (60:30:5). The desired band was eluted with methanol and the eluates were evaporated *in vacuo*. The residue was again purified by gel chromatography on Sephadex LH-20 (1.9×100 cm) eluted with methanol. The desired fractions were collected and eluates evaporated *in vacuo* to yield **4** as colorless crystals; 79 mg (41%). Mp 171—179 °C (decomp); R_f^6 0.19, R_f^7 0.22, R_f^8 0.53.

N-*(t-Butoxycarbonyl)-*L*-tyrosyl-*D*-alanylglycine Ethyl Ester (Boc-L-Tyr-D-Ala-Gly-OEt) (**21**).* Mpt-Cl (0.257 g, 2 mmol) was added to an ice-cold stirred solution of Boc-L-Tyr-OH (0.563 g, 2 mmol) and triethylamine (0.28 cm^3, 2 mmol) in chloroform (3 cm^3), after which the mixture was stirred for 1 h. To this solution a mixture of TFA·H-D-Ala-Gly-OEt (0.576 g, 2 mmol) and triethylamine (0.56 cm^3, 4 mmol) in *N,N*-dimethylformamide (2 cm^3) and chloroform (2 cm^3) was added in several portions at 0 °C. After having been stirred at this temperature for 1 h, the solution was washed in the usual way, dried and evaporated *in vacuo*. Solid residue was purified by preparative silica-gel thin-layer chromatography using chloroform–methanol (37:3) for development. The desired band (R_f=0.4—0.6) was eluted with chloroform–methanol (9:1) and eluates evaporated *in vacuo*. The residue was crystallized from hot ethyl acetate to give colorless crystals; 0.674 g (77%). Mp 164—166 °C; R_f^2 0.56, R_f^3 0.45.

N-*(t-Butoxycarbonyl)-*L*-tyrosyl-*D*-alanylglycine (Boc-L-Tyr-D-Ala-Gly-OH) (**22**).* To an ice-cold solution of **21** (0.479 g, 1.1 mmol) in methanol (3 cm^3), a 1 M aqueous NaOH solution (2.2 cm^3) was added and the solution was stirred at

0 °C for 30 min and at room temperature for 4 h. After removal of methanol *in vacuo*, the solution was acidified to pH 3 by solid citric acid at 0 °C. To this Diaion HP-20 resin was added until no spot corresponding to the product was detectable on TLC. The resin was filtered and washed with water until the filtrate became neutral. Elution of the resin with methanol and evaporation *in vacuo* of the eluates gave a chromatographically homogeneous amorphous solid; 0.430 g (95%). R_f^5 0.50, R_f^6 0.22.

N-(t-Butoxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine p-Methoxybenzyl Ester (Boc-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys(Mpt)-OMob) (23). To a mixture of **22** (0.390 g, 0.742 mmol), *N,N*-diisopropylethylamine (0.129 cm³, 0.742 mmol) and **14** (0.468 g, 0.742 mmol) in chloroform (3 cm³) and *N,N*-dimethylformamide (3 cm³), dicyclohexylcarbodiimide (0.153 g, 0.742 mmol) and 1-hydroxybenzotriazole (0.100 g, 0.742 mmol) were added at 0 °C. After the mixture was stirred at room temperature overnight, all the solvents were removed *in vacuo*. To the solid residue, dichloromethane was added, and precipitates were removed by filtration and washed. The filtrate and washings were washed as usual, dried and evaporated to give an amorphous solid. This was purified by preparative silica-gel thin-layer chromatography, using chloroform–methanol (9:1) as a developing solvent. The desired band (R_f=0.5—0.7) was eluted with methanol and eluates were evaporated *in vacuo*. The residue was dissolved again in a small volume of methanol and this mixture was placed in a Sephadex LH-20 column (1.9×100 cm), which was eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to give colorless crystals; 0.560 g (76%). Mp 141—145 °C; R_f^3 0.60, R_f^4 0.18. Found: C, 57.63; H, 6.70; N, 8.60%. Calcd for $C_{47}H_{65}N_6O_{11}PS_2$: C, 57.30; H, 6.65; N, 8.53%.

L-Tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucyl-S-dimethylphosphinothioyl-L-cysteine (H-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys(Mpt)-OH) (24). Trifluoroacetic acid (3 cm³) was added to an ice-cold solution of compound **23** (0.200 g, 0.203 mmol) in dichloromethane (2 cm³) containing anisole (0.3 cm³). After having been kept at room temperature overnight, the solution was evaporated *in vacuo*. The solid residue was washed with ether and purified by preparative silica-gel thin-layer chromatography using chloroform–methanol–29% aqueous ammonia (60:30:5). The desired band (R_f=0.3—0.5) was dissolved again in a small volume of acetic acid and the mixture was placed in a Sephadex LH-20 column (1.9×100 cm), which was eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to give **24** as colorless crystals; 0.0991 g(63%). R_f^6 0.56, R_f^7 0.47. Found: C, 52.62; H, 6.57; N, 10.45%. Calcd for $C_{34}H_{49}N_6O_8PS_2 \cdot CH_3OH \cdot 1/4H_2O$: C, 52.45; H, 6.72; N, 10.49%.

Bis(N-(t-butoxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucyl)-L-cystine Bis(p-methoxybenzyl Ester) (Boc-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys-OMob)₂ (25). To a solution of compound **23** (0.201 g, 0.204 mmol) in methanol (4 cm³) and acetonitrile (4 cm³), KF (0.0237 g, 0.407 mmol) and 18-crown-6 (0.108 g, 0.407 mmol) were added. After having been stirred at room temperature for 9 h, the mixture was treated with 0.1 M iodine–potassium iodide solution in 90% methanol (2.3 cm³) and evaporated *in vacuo*. The residue was dissolved in ethyl acetate and water. The organic layer was separated and washed with water, a sodium thiosulfate solution, water, a 5% citric acid solution, water and a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by preparative silica-gel thin-layer chromatography by using chloroform–methanol (9:1). The desired band (R_f=0.5—0.6) was eluted with methanol and the eluates were evaporated *in vacuo*. The residue was further purified by preparative silica-gel thin-layer chromatography using chloroform–methanol (95:5) and gel chromatography on Sephadex LH-20 (1.9×100 cm) eluting with methanol. The desired fractions were collected and the eluates were evaporated *in vacuo* to yield **25** as colorless crystals; 0.136 g(74%). Mp 195—198 °C; R_f^3 0.55, R_f^4 0.07. Found: C, 58.83; H, 6.64; N, 9.14%. Calcd for $C_{90}H_{118}N_{12}O_{22}S_2 \cdot 3H_2O$; C, 58.80; H, 6.80; N, 9.14%.

Bis(L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-leucyl)-L-cystine (H-L-Tyr-D-Ala-Gly-L-Phe-L-Leu-L-Cys-OH)₂ (26). Method A: To a solution of compound **24** (0.136 g, 0.178 mmol) in methanol (10 cm³) and acetonitrile (10 cm³), KF (0.124 g, 2.135 mmol) and 18-crown-6 (0.282 g, 1.067 mmol) were added. The mixture was stirred at 50 °C for 9 h and then evaporated *in vacuo*. The residue was dissolved in water (20 cm³) and to this Diaion HP-20 resin was added. The resin was filtered and washed with water (200 cm³). Elution of **26** was performed by methanol and eluates were evaporated. The residue was subjected to preparative silica-gel thin-layer chromatography using chloroform–methanol–water (65:25:4). The desired band (R_f=0.2—0.3) was eluted with methanol and the eluates were evaporated *in vacuo*. The residue was dissolved in a small volume of methanol and placed in a Sephadex LH-20 column (1.9×100 cm), which was eluted with methanol. The desired fractions were collected and evaporated *in vacuo* to give **26** as colorless crystals; 55.3 mg(46%). R_f^6 0.25, R_f^7 0.07, R_f^8 0.54.

Method B: Compound 25 (0.129 g, 0.072 mmol) was dissolved in TFA (4 cm³) containing anisole (0.4 cm³). The solution was stirred at 0 °C for 4 h and evaporated *in vacuo*. The residue was triturated with ether to give crystals, which were dissolved in a small volume of methanol and subjected to preparative silica-gel thin-layer chromatography using chloroform–methanol–29% aqueous ammonia (60:30:5). The desired band was eluted with methanol and the eluates were evaporated *in vacuo*. The residue was again dissolved in a small volume of methanol and water and placed in a column (1.2×20 cm) of Dowex 1×2 ion-exchange resin (acetate form), which was eluted with a 1% acetic acid solution. The eluates were evaporated and the residue was purified by gel chromatograhy on Sephadex LH-20 (1.9×100 cm), eluting with methanol. The desired fractions were collected and evaporated *in vacuo* to give colorless crystals; 44.5 mg (45%). Mp 198—202 °C (decomp); R_f^6 0.25, R_f^7 0.06, R_f^8 0.56. Found: C, 53.52; H, 6.22; N, 11.49%. Calcd for $C_{64}H_{86}O_{16}N_{12}S_2 \cdot 1/2H_2O$: C, 53.68, H, 6.21; N, 11.38%.

The authors wish to thank Professor Tetsuo Oka (Tokai University) for the biological activity measurements and for his encouragement throughout this work.

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